

REMARKS

Status of the Claims

Claims 1-2, 5-7, 9, 13-18, 21-22, 24, and 26 are pending in the present application. Claim 7 is amended. Claims 3, 4, 8, 10, 11, 12, 19, 20, 23, and 25 were previously canceled. Claim 15, 17, 22, and 24 are withdrawn as being directed to a non-elected invention. Claim 7 is amended to clarify that the human native protein has the described sequence identity to a homolog protein "over the whole length" of the mouse to be immunized. Claim 26 is new. Support for new claim 26 is found, for example, in pending claims 5 and 6. No new matter is entered by way of this amendment. Reconsideration is respectfully requested.

Issues Under 35 U.S.C. § 102(b)

Claims 7, 9, 14, 16, and 18 are rejected under 35 U.S.C. § 102(b) as allegedly being anticipated by U.S. Patent No. 6,235,714 to Paul *et al.* ("Paul") for the reasons set forth in the Office Action of December 5, 2008, *see Office Action*, pages 2-3. In the December 5, 2008, Office Action, the Examiner stated that Paul teaches that the human antigen in the exemplary CRAA-IL1- β peptide, (PKKKMEK) has 100% identity at the amino acid sequence level to the mouse protein antigen, *see page 7 of the December 5, 2008, Office Action*. In addition, the Examiner stated that "[g]iven that the claim does not specify the length of the amino acid level... PKKKMED reads on the claimed invention", *see page 7 of the December 5, 2008, Office Action*. Applicants respectfully traverse.

Although Applicants do not agree with the Examiner, independent claim 7 is amended in an effort to expedite prosecution. As amended, claim 7 is directed a process for producing an antibody comprising immunizing a mouse with Fas function defects with a human native protein, wherein said human native protein has a sequence identity of 94% or more at the amino acid sequence level to a homolog protein over the whole length, of the mouse to be immunized.

Applicants submit that the antigens described in Paul are not encompassed by the human native proteins described in independent claim 7. As Applicants noted previously, the claims specify native proteins and native proteins do not encompass the PKKKMEK sequence. In addition, independent claim 7, as amended, does not encompass the antigen fragments as

allegedly described in Paul, since the claims describe “a human native protein which has a sequence identity of 94% or more at the amino acid level to a homolog protein over the whole length of the mouse to be immunized.”

In view of the foregoing, independent claim 7 is not anticipated by Paul. Further, dependent claims 9, 14, 16, and 18, which incorporate all of the elements of independent claim 7, are also not anticipated by Paul. Accordingly, withdrawal of the rejection is respectfully requested.

Issues Under 35 U.S.C. § 103(a)

Mashiko, Yamasaki, Makino, and Lage

Claims 1-2, 5-7, 9, 13-14, 16, and 18-21 are rejected under 35 U.S.C. § 103(a) as allegedly obvious over JP-01047390 to Yamazaki Mashiko *et al.*, (“390”) or U.S. Patent No. 4,965,198 to Yamasaki *et al.*, (“198”) each in view of Makino *et al.*, *J. Clin. Lab. Immunol.*, 1988, 25:83-88, (“Makino”), and Lage *et al.*, *Virchows Arch*, 2001, 438:567-573, (“Lage”), *see Office Action*, pages 3-6. Applicants respectfully traverse.

Specifically, the Examiner states that the ‘390 publication discloses NZB, NZW, B/WF1, MRL/I, BXSB male, and SLTN1 mouse strains. The Examiner further states that the ‘198 patent discloses NZB, NZW, B/WF1, MRL/1, BXSB (SLE) male and SLNi autoimmune mice strains. In addition, the Examiner states that Makino teaches that comparative studies between male BXSB and MRL/lpr mice at the onset period show that MRL/lpr mice have much higher levels of serum immune complexes (IC) than male BXSB mice at 13 weeks, as assessed by fluid- and solid-phase C1q-binding assays. The Examiner concludes that an ordinary artisan would have been motivated to use MRL/lpr mice because MRL/lpr mice would have been known to produce much higher levels of serum IC than male BXSB mice. Lage is cited for describing that GPC3 is weakly immunogenic in mice.

Initially, Applicants submit that BXSB mice were not elected in response to the species election requirement, *see* March 31, 2008, Office Action and response of September 29, 2008. Applicants further submit that the BXSB mouse, similarly to the MRL/lpr mouse, exhibits an immunodeficient phenotype as a lupus prone mouse. Accordingly, Applicants believe that it is

unreasonable to judge whether or not the instant claims are unobvious by assessing the capability of MRL/lpr mice to produce antibodies in view of antibody production of BSKB mice.

Makino teaches (i) that there is no difference between the degradation of IC in the glomus between BXSB mice and MRL/lpr mice. Degradation of IC in the glomus is believed to cause lupus glomerulonephritis (LGN), a disease that both BXSB and MRL/lpr mice suffer from. Makino further teaches (ii) that the level of IC, which binds to Raji cells, is not different between BXSB mice and MRL/lpr mice. The Examiner notes that BXSB mice and MRL/lpr mice differ in the level of IC binding to Clq and concludes that an ordinary artisan would have believed from this observation that MRL/lpr mice are preferable. However, Applicants submit that the Examiner is using improper hindsight in view of the claimed invention to make this conclusion. As noted above, Makino discloses that there is no difference in IC degradation in the glomus between the mice strains. Further, Makino discloses that the levels of IC binding to Raji cells is not different between the two strains. Accordingly, Applicants submit that an ordinary artisan, reviewing Makino as a whole, would not have come to the Examiner's conclusion that an ordinary artisan would have been motivated to use MRL/lpr mice to achieve the instant invention.

In further support of this position, Applicants submit herewith Tomer *et al.*, *Immunological Investigations*, 1988, 17(5):389-424, ("Tomer *et al.*"), which cites numerous journal articles. Tomer *et al.* teach that autoimmune antibodies, such as anti-DNA and anti-rheumatoid factor antibodies, are immediately generated when a B cell activator, such as bacterial lipopolysaccharide (LPS), is administered to a normal mouse, *see* page 398, line 5 to page 399, line 9 of Tomer *et al.* Tomer *et al.* further show that a normal mouse, such as Balb/c mouse, activated by LPS and the like, is equally capable of producing autoantibodies as an immune deficient mouse, such as MRL/lpr, *see* page 400, lines 3-24 of Tomer *et al.*

In the June 4, 2009, response, Applicants insisted that the '390 application and the '198 patent teach that not only do immune deficient mice have an improved ability to produce autoantibodies, but Balb/c mice, which have been administered a B cell activator, such as LPS, may also be immunized. This knowledge is based on technical common sense and is discussed in Tomer *et al.* Moreover, many other references show that Balb/c mice, which are activated by

a B cell activator, can be used as immunized animals. For example, U.S. Patent No. 4,942,131 teach that Balb/c mice, which have been administered a B cell activator such as LPS, have an improved ability to produce autoantibodies, and can be used as an immunization animal to produce anti-GM3 (4-O-Ax-NeuGc) antibodies, *see* column 4, line 58 to column 5, line 6.

Accordingly, an ordinary artisan would not have recognized from the cited references that a mouse, such as an MRL/lpr mouse, which lacks Fas function, is more preferable than a Balb/c mouse, which is administered LPS and the like, for antibody production against a human antigen, which has a high sequence identity at the amino acid sequence level to a homolog protein over the whole length, of the animal to be immunized. In fact, an ordinary artisan reviewing the cited references would have concluded quite the contrary. An ordinary artisan would have recognized that the ability to produce autoantibodies from a mouse with an autoimmune disease would have been similar to a Balb/c mouse, which has been administered LPS or the like. As stated in the previous response, it is Applicants' position that an ordinary artisan would have been reluctant to use a mouse having an autoimmune disease to produce the described antibodies if the ordinary artisan was aware at the time of the invention that there is no advantage to using such a mouse when a Balb/c mouse, modified as described above, may be used. In this respect, Applicants reiterate that the cited references teach away from the claimed invention.

In view of the foregoing, Applicants submit that the claims are not obvious in view of the combination of cited references. Withdrawal of the rejections is respectfully requested.

Mashiko, Yamasaki, Lage and Wysocki

Claims 1-2, 5-7, 9, 13-14, 16, and 18-21 are rejected under 35 U.S.C. § 103(a) as allegedly being obvious over the '390 publication or the '198 patent, each in view of Lage and further in view of U.S. Patent No. 5,641,488 to Wysocki ("488"), *see Office Action*, pages 10-11. Applicants respectfully traverse.

As noted above, it is Applicants' position that the '390 publication, the '198 patent and Lage teach away from the instant invention. Accordingly, the claims are not obvious in view of these references. The '488 patent is merely cited for teaching MRL/lpr animals. Accordingly,

the '488 patent does not remedy the deficiencies of the '390 publication, the '198 publication and Lage.

Based upon the foregoing, the claims are not obvious over the cited references. Accordingly, Applicants respectfully request withdrawal of the rejection.

CONCLUSION


In view of the above, Applicants believe that the pending application is in condition for allowance.

Should there be any outstanding matters that need to be resolved in the present application, the Examiner is respectfully requested to contact Linda T. Parker, Reg. No. 46,046, at the telephone number of the undersigned below, to conduct an interview in an effort to expedite prosecution in connection with the present application.

If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies to charge payment or credit any overpayment to Deposit Account No. 02-2448 for any additional fees required under 37.C.F.R. §§1.16 or 1.17; particularly, extension of time fees.

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THE SIGNIFICANCE OF

NATURAL AUTOANTIBODIES

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ABSTRACT

Since Burnet first introduced his "forbidden clones" theory, the discrimination between self and non-self and the physiologic mechanisms of avoiding autoimmunity remained an enigma. The realization in the past two decades that autoantibodies reacting with various self antigens are common in normals has led to intensive research on the origin and physiologic role of these "natural autoantibodies". After reviewing the extensive literature on the appearance of natural autoantibodies in normal animals and humans, and the studies proving unequivocally that natural autoantibodies are coded by germ line genes, we will discuss the current hypotheses explaining their appearance and physiologic role. Despite the fact that numerous hypotheses explaining the origin of natural autoantibodies have been postulated only the two important ones will be discussed. The first, proposed by Cunningham, holds that clonal deletion as viewed by Burnet operates in early life; however, later in life all autoreactive B cells not eliminated during ontogeny are prevented from expanding and secreting anti-self antibodies by a compensatory suppressor mechanism. Therefore, natural autoantibodies are postulated to be autoantibodies which are produced only in minute quantities allowed by the suppressor mechanism. The second hypothesis views autoantibody formation as a result of cross reaction between foreign and self determinants. It is suggested that the part of the B cell population which gives rise to autoantibodies carries a polyspecific receptor; fixation of a foreign antigen to this receptor induces the B cell to undergo a series of divisions and mutations, which under the selective pressure of the antigen leads to production of a highly specific antibody. Thus natural

autoantibodies may constitute the antibodies secreted by these B cells prior to encountering foreign antigens. The biologic role of natural autoantibodies is also elusive. The common denominator to all the theories dealing with that puzzling question is the view that natural autoantibodies have a positive role in normal immune reactions, perhaps even an essential role without which normal immune function would be disrupted. Grabar suggested that natural autoantibodies are part of a physiologic mechanism for cleansing the organism of self and non-self products in which classical antibodies serve to clear the body of foreign invading agents, while natural autoantibodies rid the organism of its own catabolic products. Cohen and Cooke suggested that natural autoantibodies, by binding to self antigens, act as a filter preventing powerful immune response against self triggered by self mimicking determinants on foreign invading microorganisms. Others have suggested a role for natural autoantibodies in the idiotypic network. We propose a different function for natural autoantibodies, namely enhancing host immune reactions to foreign infectious agents, in a similar manner in which MHA antigens participate in the activation of B and T cells. The question of the origin and biologic role of natural autoantibodies is not a purely academic one, and understanding these mechanisms will certainly clarify the pathogenesis of autoimmune diseases and autoimmunity in general.

INTRODUCTION

The fundamental basis of the immune response is the discrimination between self and non-self. This primary and most essential feature of the immune system enables it to recognize

and react to foreign invading agents without causing self damage. However, as our knowledge of immune responses grew, it was realized that various antibodies reactive with self constituents can be synthesized by the normal immune system. Realizing that autoantibodies appear under certain conditions, Ehrlich coined his famous term "horror autotoxicus", expressing the view that autoimmunity is always a pathological process, usually with grave clinical consequences. The first theory dealing with the physiologic ways of avoiding autoimmunity was postulated by Burnet [1]. It stated that all anti-self lymphocytes are eliminated in embryonic life; these autoreactive lymphoid cell lines were termed "forbidden clones", denoting the necessity to eradicate them in order to prevent "horror autotoxicus". The mechanism proposed for the elimination of the forbidden clones assumed that all lymphocytes responding to antigenic challenge during ontogeny were destroyed; therefore, in fetal life all clones of lymphocytes encountering and responding to autoantigens are eliminated before maturation. According to this theory autoantibodies, which are always pathologic, arise as a consequence of emergence of forbidden clones by mutations, or of liberation of sequestered antigens. As we shall see later, a growing body of evidence contradicting this theory has accumulated. To name a few examples: various autoantibodies can be detected in normal healthy subjects [2-9], lymphocytes from normal mice and humans can be stimulated *in vitro* to produce autoantibodies [6,10,11], autoantibodies can be induced *in vivo* in normal mice [12-15], and hybridomas can be formed between normal mice or human lymphocytes and non-secreting myeloma cell lines which produce autoantibodies [5,16-18,51]. In light of the substantial data showing that autoimmune

reactions are common in humans and animals with normal functioning immune system, new theories were proposed to explain this "paradoxical" phenomenon. In the present review we shall outline the evidence for the existence of natural autoantibodies and the mechanisms proposed to explain their emergence, as well as discuss their physiological role.

NATURAL ANTIBODIES

Before proceeding to discuss natural autoantibodies (NAA's), a note must be made on natural antibodies (NA's) in general. The existence of NA's in normal human serum was first reported by Landsteiner [100] when he found in the serum natural hemagglutinins directed against blood group determinants of the ABO system. NA's were first defined as antibodies found in the serum of animals that have not been previously immunized. The original explanations for their appearance viewed unsuspected antigenic challenge as the main cause. However, today many workers believe that NA's are formed independently of antigenic stimulation [19]. Therefore, NA's were redefined as antibodies found in the serum and which do not require induction of B cells (such as by antigenic challenge or mitogenic stimulation) for their synthesis. Moreover, NA's are viewed as a part of natural immunity which is a component of the immune system serving as a first line of defence, and capable of destroying foreign agents on initial contact [19]. The main components of natural immunity include macrophages, natural killer cells and natural antibodies. The role of NA's in natural resistance is thought to be in favoring a more vigorous host response to pathogens, and indeed NA levels were found to correlate with host resistance to

infection [20,21]. Some workers believe that NA's are actually the active mediators of natural immunity and that they are markers of disease outcome [20,22]. As will be outlined in detail later, in the past two decades NA's reacting with various autoantigens were found to be very common [2-9]. Therefore, a clear distinction between NA's and NAA's cannot be made.

AUTOANTIBODIES IN SPECIAL POPULATIONS

a) First degree healthy relatives of patients with autoimmune conditions:

The first evidence for the existence of autoantibodies in healthy individuals came from studies done in relatives of patients with autoimmune diseases. A significant proportion of asymptomatic first degree relatives of SLE patients were found to have anti-nuclear antibodies (ANA) [23], anti-DNA antibodies [24], rheumatoid factor [25], anti-histone antibodies [26], and lymphocytotoxic antibodies [27]. Isenberg and colleagues [28] found two public anti-DNA antibody idiotypes (16/6 and 32/5) in 24% and 7% respectively of healthy relatives of SLE patients. The sharing of these idiotypes by antibodies obtained from different patients and their healthy relatives suggests that these hypervariable region structures are the product of germ line genes present in lupus patients and their asymptomatic family members. As we shall see later these genes are widely dispersed in the general population. Autoantibodies were also found in asymptomatic relatives of patients with other autoimmune diseases. High incidence of rheumatoid factor was reported in healthy first degree relatives of patients with

rheumatoid arthritis [29]. Similarly, thyroid autoantibodies were found in symptom-free relatives of patients with autoimmune thyroid disease [30], islet-cell antibodies were found in a small percentage of first degree relatives of patients with insulin dependent diabetes mellitus [31], and anti-centromere antibody was found in 3.5% of relatives of patients with scleroderma [32].

b) Ageing and natural autoantibodies:

Another important segment of the healthy general population, frequently showing autoantibodies in their serum, are aged people (for a recent review see [90]). Since the first descriptions of the high frequencies (up to 42%) of IgM-RF found in aged individuals [33-35], increased serum levels of many other autoantibodies were reported in elderly subjects. Notable are the increased frequencies of ANF and anti-thyroglobulin antibodies described in elderly people [34,37]; moreover, in vitro studies in spleen cells from old mice demonstrated substantial production of auto-anti-red blood cell (RBC) antibodies by these cells [38].

As a consequence of the above described findings, the question arises whether the autoantibodies found in apparently healthy people arise as a result of genetic or environmental factors. At first, the hypothesis attributing the formation of autoantibodies to environmental factors seemed attractive. DeHoratius and Messner [27] found increased incidence of lymphocytotoxic antibodies in both consanguineous and nonconsanguineous relatives of lupus patients, supporting a role for an environmental factor (virus?) in the pathogenesis. Others [39] described a correlation between titers of anti-rheumatoid arthritis nuclear antigen (RANA) and anti-Epstein-Barr nuclear

antigen (EBNA) antibodies in normal adults, suggesting that anti-RANA antibodies appear only after prior EBV infection. Moreover, Welch et al. [8] have shown that tetanus toxoid vaccination of normal adults caused a 2-3 fold rise in the frequency of IgM-RF precursor B cells. These findings suggest a role for foreign agents (such as infectious organisms) in the formation of autoantibodies. However, even if we accept the view that autoantibodies are produced, at least in some cases, as a result of foreign antigens, the mechanism is most likely to be polyclonal B cell activation inducing autoreactive B cells to produce autoantibodies. The other possible mechanism of specific B cell activation by cross reaction between the foreign antigenic determinants and self determinants or by adjuvant-like action of the invading agent has been shown to be improbable [40].

c) Natural autoantibodies among immunoglobulins in plasma cell dyscrasias:

Monoclonal gammopathies (multiple myeloma, Waldenström's macroglobulinemia) arise from the expansion of one clone of lymphocytes. At first, monoclonal immunoglobulins (M-Ig's) produced by monoclonal gammopathies were considered to be abnormal immunoglobulins lacking antibody activity. However, in the past two decades it has been shown that a large proportion of M-Ig's have normal antibody activity when tested against various antigens. A striking finding was that a significant portion of the M-Ig's are directed against self determinants. Avrameas et al. [5] found in 5.7% of 612 M-Ig's tested antibody activity against 5 common autoantigens (actin, tubulin, thyroglobulin, dsDNA and myosin). Other monoclonal antibodies were also found to react with IgG [42,43], RBC, fibrin,

transferrin, albumin, cardiolipin, heparin, and DNA [44-48]. In most cases there were no signs of an autoimmune disease. Furthermore, Shoenfeld et al. [49] reported recently increased concentrations of the common anti-DNA idiotype 16/6 id. in 8.7% of 255 monoclonal antibodies tested; in 1.9% of the cases anti-nuclear activity was also present. The studies on patients with monoclonal gammopathies raised the important question of the underlying mechanism involved in the production of autoreactive M-Ig's. One possibility is that B lymphocytes, synthesizing autoantibodies are more susceptible to neoplastic transformation. Another explanation is that B cells producing autoantibodies are very common. Indeed, new substantial findings demonstrate almost unequivocally that autoreactive lymphocytes are part of the normal B cell population and that autoantibodies are commonly produced in normal individuals. Therefore, we shall refer to these autoantibodies as natural autoantibodies (NAA's).

To date substantial evidence for the existence of NAA's in normal animals and humans has accumulated. We will begin by reviewing the animal studies.

NATURAL AUTOANTIBODIES IN ANIMALS

There are several approaches to the study of autoantibody production in animals; the level of NAA's can be measured in the serum and these autoantibodies compared to those secreted by strains of mice spontaneously developing autoimmune diseases, the level of autoantibody production can be determined after inn

vivo stimulation by mitogens, or after in vitro stimulation of spleen cells. Finally, normal mice spleen cells can be fused with non-secreting myeloma cell lines and the antibodies produced by the hybridoma studied.

NAA's were reported in the serum of normal mice by many workers. These include anti-DNA antibodies, found in several normal strains of mice [41], thymocytotoxic antibodies [62], anti-IgG antibodies [61], and antibodies to elastin, collagen, gelatin, RBC's and thyroid cells [60]. Moreover, injection of polyclonal B cell activators (PBA's) caused rapid synthesis of NAA's. Injection of lipopolysaccharide (LPS) into normal mice resulted in production of anti-DNA antibodies [12,64], rheumatoid factor [65,66], anti-RBC antibodies [14,57], and anti-thymocyte antibodies [15]. Striking findings were reported by Dziarski [13], who found that the majority (about 60%) of immunoglobulin secreting spleen cells in LPS and peptidoglycan (PG) injected mice secreted rheumatoid factor antibodies; less than 3% of the spleen cells secreted anti-DNA antibodies. These results were similar in 3 different strains of mice, suggesting that there is no H-2 restriction, and that it is a general phenomenon.

It is well known that LPS injection causes a rapid release of DNA into the blood [64,78]. Therefore, induction of anti-DNA antibodies by PBA's (mainly by LPS) may result from antigenic stimulation of the DNA released into the circulation rather than from polyclonal activation of B cells committed to produce anti-DNA antibodies. Brilliant studies by Izui and colleagues [12,68] proved that the mechanism is not antigenic stimulation by DNA released into the blood. Izui et al. have shown that there was a complete dissociation between the formation of anti-DNA

antibodies and the appearance of DNA in the blood (e.g. PPD, a known polyclonal B cell activator (PBA), induced anti-DNA antibody production without causing release of DNA into the blood, and vice versa: poly I-poly C did not trigger production of anti-DNA antibodies although it induced release of DNA into the circulation). These results strongly suggest that the induction of anti-DNA antibodies by PBA's is a consequence of polyclonal activation of B lymphocytes committed to produce anti-DNA antibodies. Furthermore, Madala and co-workers [40]

have shown that autoimmune strains of mice do not respond to immunization with native DNA while normal mice do but produce antibodies different than NAA's. When these mice were immunized with denatured DNA, autoimmune mice (MRL++) did not respond while normal mice (C57BL/6) did; the induced anti-nucleic acid antibodies were highly specific and reacted only with the immunogen. In contrast both lupus autoantibodies and sera from animals that received adjuvant alone cross reacted with multiple nuclear antigens. These results again show that polyclonal B cell activation is the mechanism of induction of autoantibodies by PBA's in normal mice, and suggest that polyclonal B cell activation may be the mechanism of development of autoimmune diseases, perhaps occurring as a consequence of failure of the inhibitory mechanisms (T suppressor cells?). Indeed, neonatal thymectomy in normal mice was shown to induce accelerated anti-DNA antibody production after PBA injection [69]; when thymectomy was combined with PBA treatment, a much greater amount of anti-DNA was produced than when PBA was given alone. These results suggest that a thymic regulatory process normally serves to suppress anti-DNA production. Supporting this view is the finding in the same study [69] of deficient antigen non-

specific suppressor function in neonatally thymectomized and PBA treated mice.

In vitro studies in which mouse spleen cells were activated by PBA's gave results similar to those reported in the in vivo studies, thus lending further support to the view that autoreactive B cells are part of the normal lymphocytic repertoire. Using in vitro studies, several workers were able to estimate the relative proportion of autoreactive cells in the splenic lymphocyte population. Despite the differences between reports of different authors, the hallmark of these studies is the surprising finding that autoreactive lymphocytes constitute a large portion of the splenic antibody producing cell population. Dziarski [11] studied the specificities of antibodies produced by polyclonally activated mouse spleen cells (PBA's used were LPS and peptidoglycan [PG]), and reported that 41%-75% of all PG and LPS activated IgM secreting cells produced anti-IgG antibodies of the IgM class. A smaller proportion produced anti-ssDNA and anti dsDNA antibodies (4.4%-17% and 0.18%-5.9%, respectively). Other workers reported similar results [70,71,98]. Pisetsky and Caster [70] enumerated the frequency of precursors of anti-DNA antibody producing cells in normal (BALB/C, BA/J) and autoimmune (MRL-lpr/lpr) mice, and found that approximately 1 in 500 spleen cells from both normal and autoimmune mice were capable of producing anti-DNA antibodies.

Similarly, Conger et al. [98] have demonstrated that 1-5% of the total mitogen-induced antibody forming cell (AFC) clones derived from spleens of normal, autoimmune, and congenitally athymic mouse strains secreted anti-denatured DNA (ssDNA) antibodies. These results again indicate that B cells from spleens of normal mice have the potential to express autoantibodies. Neither the

autoimmune strain derived splenocytes nor the congenitally athymic strain splenocytes have shown greater frequency of autoreactive precursors than normal mice. Therefore, it appears that autoimmune disease is not the result of preferential expansion of this precursor cell population causing production of autoantibodies but rather is associated with alteration in their state of activation. The high frequency of anti-DNA precursors suggests why B cell activation so readily leads to anti-DNA production.

The hybridoma technique of producing monoclonal antibodies was used extensively in NAA research, and helped to unravel some of the uncertainties surrounding the subject. Following their experiments in sera from healthy donors (see below) and human monoclonal immunoglobulins, Avrameas and co-workers [5] continued and tested hybridomas produced by fusion of splenocytes from non-autoimmune mice (BALB/C) with non-secreting myeloma cell line for their antibody activity. The immunoglobulins produced by these hybridomas were tested for their activity against 11 common antigens, namely actin, tubulin, myosin, thyroglobulin, myoglobin, spectrin, dsDNA, fetuin, TNP, transferrin and GAT. Of the 161 immunoglobulin secreting hybridomas, three reacted with dsDNA only, one with thyroglobulin only, and another reacted mainly with myosin. Therefore, 5 hybridomas (3.1%) produced autoreactive antibodies. These results support the notion that autoreactive immunoglobulin-synthesizing clones exist in normal mice (and probably humans). By the same technique, hybridomas prepared by fusion of spleen cells from normal non-autoimmune mice with non-secreting myeloma cell lines were shown to produce autoantibodies against numerous other autoantigens (e.g. pancreas, pituitary, stomach [16], and thyroglobulin [18]).

Anti-DNA antibodies were demonstrated in several studies using hybridomas [17,18]. These anti-DNA antibodies exhibited a widespread reactivity similar to monoclonal lupus autoantibodies [85], but in contrast to the high specificity of induced antibodies obtained after active immunization [18]. This suggests a different origin and role for NAA's than for induced antibodies. In summary, the hybridoma studies, like the in vivo and in vitro polyclonal activation studies, support the notion that B cells with genes coding for autoantibody synthesis are present in normal mice.

An important contribution to the theory that germ line genes coding for autoantibodies exist in all animals and humans comes from the studies of Dighiero et al. [72] in newborn mice. They were able to show the existence of B cells capable of producing autoantibodies in newborn normal mice. Spleen cells from 5 day old non-immunized BALB/C and BALB, B/O mice were fused with a non-secreting myeloma cell line. Out of 384 immunoglobulin secreting hybrids, 24 (6.25%) exhibited antibody activity against a panel of autoantigens tested (mouse actin, tubulin, myosin, renin, DNA and more).

The extent of autoreactivity demonstrated in animals certainly contradicts Burnet's theory [1] that autoreactive B cell clones are eliminated during ontogeny. In light of these findings we can conclude that autoantibodies are part of the normal antibody repertoire, and that most probably genes coding for their synthesis are an inherent part of the normal germ line cell genome. Further evidence favoring the existence of germ line genes coding for autoantibodies comes from studies in humans.

NATURAL AUTOANTIBODIES IN HUMANS

The evidence which served to substantiate the theory that autoreactive lymphocytes exist and secrete autoantibodies in normals came from human studies. It has long been known that all normal sera contain a panagglutinin reacting with RBC's previously treated with proteases [50]. The formation of this first discovered NAA did not seem enigmatic since its role - opsonizing senescent RBC's in order to induce their phagocytosis - appeared natural. However, as more and more NAA's were described, and it was realized that a large diversity of AA's are synthesized in normal individuals, their origin became obscure. A summary of the important reports of NAA formation in humans is presented in table no. I. The striking conclusion from these reports is that NAA's reacting with almost any antigen tested can be found; these include autoantibodies usually found in autoimmune diseases, but also autoantibodies which do not appear in pathological states.

The appearance of anti-DNA antibodies in normal individuals has been reported by several workers [2,6,10,51-53]. Rubin and Carr [2] have recently shown that normal human serum contains a small fraction of IgG capable of binding DNA. The binding activity was largely restricted to denatured DNA (ssDNA) and was shown to involve the IgG (Fab')₂ fragment. Other studies have shown anti-DNA antibody synthesis (against either ssDNA or dsDNA) after activation of peripheral blood mononuclear cells (PBMC's) by polyclonal B cell activators (PBA's). Anti-DNA antibody formation was demonstrated after stimulation of lymphocytes by PWM [54], EBV [52], *K. pneumoniae* [10], and even without mitogenic stimulation [6,53]. A notable example of the

TABLE NO. I

Reports on the presence of autoantibodies in healthy subjects.

AUTOANTIBODY	NO. OF SUBJ.	% WITH AA	TYPE OF STUDY PERFORMED	REF
RF	121	42-46%	serum from subj. over 65	86
RF	50	2 - 4%	serum from subj. under 65	86
RF	325	16%	serum from subj. over 70	87
RF	155	app. 27%	serum from subj. aged 55-98	86
RF	150	2%	serum from subj. aged 16-25	86
ANF	155	11%	serum from subj. aged 59-98	86
ANF	150	2.5%	serum from subj. aged 16-25	86
ANTI - DNA	16	app. 100%	spont. secret. tonsillar lymph.	6
ANTI - DNA	N.S.	100%	normal human serum	2
ANTI - DNA	12	25%	spontaneously secreting PMBC's	33
ANTI-DNA (16/6)	6	100%	PBA stimulated PMBC's	10
ANTI - ssDNA	6	100%	PBA stimulated PMBC's	10
ANTI - dsDNA	6	100%	PBA stimulated PMBC's	10
ANTI-BRAIN TIS.	20	100%	serum from normal subjects	3
ANTI-HEAT TIS.	20	100%	serum from normal subjects	3
ANTI-LIVER TIS.	20	100%	serum from normal subjects	3
ANTI - MYELIN	50	88%	serum from normal individuals	36
ANTI - TUBULIN	800	100%	serum pool from healthy donors	4,5
ANTI - ACTIN	800	100%	serum pool from healthy donors	4,5
ANTI-THYROGLOB.	800	100%	serum pool from healthy donors	4,5
ANTI-MYOGLOBIN	800	100%	serum pool from healthy donors	4,5
ANTI-FETUIN	800	100%	serum pool from healthy donors	4,5
ANTI-TRANSFERRIN	800	100%	serum pool from healthy donors	4,5
ANTI - ALBUMIN	800	100%	serum pool from healthy donors	4,5
ANTI-CYT. C	800	100%	serum pool from healthy donors	4,5
ANTI - COLLAGEN	800	100%	serum pool from healthy donors	4,5
ANTI-RBC (cold)	NI	app. 100%	sera from healthy persons	59
ANTI-RBC (G+I)	NI	100%	sera from normal individuals	9
ANTI-FIBROBLASTS	3	100%	lymphocytes stimulated by EBV	7
ANTI-ACROSOMAL	131	99%	sera from normal individuals	84
ANTI-NEURAL TIS.	200	99%	serum samples from healthy ind.	85

N.S. not specified.

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ability of B cells from normal persons to synthesize anti-DNA antibodies has been reported by Cairns et al. [51]. After showing that circulating and tonsillar B cells of normal donors synthesized in vitro anti-DNA antibodies [6], they produced hybridomas by fusing a human non-secreting myeloma cell line with tonsillar lymphoid cells from a normal donor [51]. Out of 110 hybridomas, 13 (11.8%) produced anti-dsDNA antibodies. One hybridoma produced a monoclonal antibody with polyspecific ligand binding properties that were indistinguishable from those of monoclonal lupus antibodies [89]. These studies imply that genes coding for anti-DNA autoantibodies similar to those produced in SLE exist in the genome of normal B lymphocytes.

Other evidence for the existence of germ line genes coding for autoantibodies in normals was reported by El-Roiy et al. [10]. They found significantly increased levels of the common anti-DNA idiotype 16/6 [89] after polyclonal activation of peripheral blood mononuclear cells (PBMC's) from normal subjects. The fact that different autoantibodies from different individuals possessed the same public idiotype implies that this V region determinant is the product of a germ line gene which may be an inherent part of the normal human genome. Indeed, it has been shown [95] that different monoclonal 16/6 positive lupus autoantibodies show structural similarity in the NH₂ terminal amino acid sequences, thereby suggesting that these autoantibodies stem from a common germ line gene. In light of these data, the findings of El-Roiy et al. [10] strongly suggest that normal B cells possess genes coding for different anti-DNA antibodies and that these genes stem from a common germ line gene.

One of the most convincing proofs that NAA's against almost any self determinant examined exist in normal human serum was

provided by Avrameas and co-workers [4,5]. They tested a serum pool from 800 healthy donors for antibodies against 9 common self antigens: tubulin, actin, thyroglobulin, myoglobin, fetuin, transferrin, albumin, cytochrome C and collagen. The serum pool tested contained all 9 autoantibodies, and they have been shown to bind specifically to the antigen via the F(ab')₂ fragment and not via the Fc portion. Moreover, the authors were able to show, using immunochemical staining methods, that these NAA's are capable of reacting with human cellular constituents. Another important finding in the same study was that these naturally occurring autoantibodies, when compared to induced antibodies by competitive and non-competitive assays, showed a different equilibrium rate, namely they reacted with an antigen at a lower rate than that of induced antibodies.

Still more convincing evidence on the widespread nature of NAA's and on their ability to react with almost any self antigen chosen comes from the studies of Daar and Fabre [3]. They examined the presence in normal sera of autoantibodies against brain, liver, heart and kidney tissues, and found that every serum tested gave positive reactions with all tissue homogenates except kidney; the autoantibodies proved to be of the IgM class. A notable finding was that sera from multiple sclerosis patients were indistinguishable from normal sera in their binding to brain homogenate. Since the self antigens used in this experiment were tissue antigens, it can be concluded that antibodies combining with these antigens in vitro do so also in vivo in normal disease-free humans.

Among the many autoreactive natural antibodies found in the past two decades are the antibodies which bind to a variety of normal cellular surface and cytoplasmic antigens. These include

antibodies against myelin [56], thymus cells [57], lymphocytes [58], RBC [9,59], cultured human fibroblasts [7] and IgG (rheumatoid factor) [8,60].

In conclusion, the human and animal studies, using various techniques, show that in normal humans and mice there exists a population of B cells committed to produce autoantibodies; this population probably constitutes a large proportion of the B cell population. The autoreactive B cells most probably arise from germ line precursors, possessing in their genome genes coding for autoantibody synthesis. In other words, these autoreactive clones are not produced by mutation or some other environmental changes, but rather constitute part of the normal B cell repertoire. Many authors believe that in the normal state there exists a regulatory system that prevents the expansion of the autoreactive clones, and that failure of this regulatory mechanism causes autoimmune disease [53,54,59,86,91]. However, the existence of autoantibodies in the serum of normal mice and humans, albeit in small amounts, shows that these autoreactive clones do mature and produce the antibodies they are programmed to secrete under normal conditions. Therefore, an important question is raised: what is the origin and biologic role of these natural autoantibodies?

THE ORIGIN OF NAA'S

Commonly, when a large number of hypotheses explaining a phenomenon are formulated it indicates that very little is known about it. Indeed, many theories have been proposed to account for the unexpected finding that autoantibodies reacting with

virtually all self antigens are formed in all normal humans (table no. II). However, none of these hypotheses is either adequate or fully comprehensive. We shall concentrate on the two prevailing theories, namely on that which advocates an immunosuppressive mechanism preventing expansion of autoreactive clones in adult life and the theory which holds that autoantibodies arise from antibodies possessing a polyspecific binding site.

The impressive data showing that natural antibodies to virtually all self antigens are present in every normal serum seriously challenged the validity of Burnet's clonal selection theory. Certainly, many "Forbidden clones" harboring genes coding for autoantibodies are not eliminated in embryonic life. Some of these clones continuously secrete AA's, albeit in small amounts, and others can easily be stimulated to synthesize AA's in vitro and in vivo. Realizing that autoreactive B cells are not eliminated during ontogeny, Cunningham [63] expanded the clonal selection theory to account for these new data. He postulated that clonal deletion operates in early life, eliminating a certain proportion of autoreactive clones, perhaps particularly harmful B cell lines (e.g. clones able to produce autoantibodies against important cellular structures). However, later in life all autoreactive B cells not eliminated during ontogeny are prevented from expanding and secreting anti-self antibodies by a compensatory suppressor mechanism. Therefore, AA's, produced only in minute quantities due to the suppressor mechanism, are NAA's; failure of this mechanism would lead to the expansion of the existing autoreactive B cell clones, the production of autoantibodies in large amounts and the development of autoimmune disease. Indeed, the small amounts of

TABLE NO. II

Current theories explaining the origin and role of NAA's

I. Origin of NAA's

- A. Burnet - emergence of forbidden clones by mutations [1].
- B. Cunningham - NAA's are AA's produced in minute quantities due to a regulatory suppressor mechanism [53].
- C. Fournie et al, - release of auto-antigens (e.g. DNA) as a result of viral, parasitic, or bacterial infections causes production of NAA's [64].
- D. Polyclonal activation of autoreactive B cells [27].
- E. NAA's are produced by B cells carrying a polyspecific receptor, which upon encountering a foreign antigen synthesizes a highly specific antibody for that antigen [5].

II. Biologic role of NAA's

- A. Grabar - NAA's are a part of a physiological mechanism for cleansing the organism of self and non-self products [79].
- B. Cohen and Cooke - NAA's act as a filter preventing auto-antigens from inducing a powerful immune response triggered by cross reacting antigens on infectious organisms. [80].
- C. NAA's are part of the idiotypic - anti-idiotypic network [36].
- D. NAA's function to enhance immune responses to foreign antigens (similar to the role of MHC receptors in T and B cell interactions).

NAA's found in contrast to the large quantities of self antigens present support the notion of a regulatory suppressor mechanism.

The most substantial evidence supporting the existence of an immunoregulatory mechanism was provided by the works of Dziarski [13]. Spleen cells from normal mice were induced to produce rheumatoid factor and/or anti-ssDNA antibodies in vivo by injecting the mice with polyclonal B cell activators (LPS and PG), or in vitro by stimulation with the same PBA's. It was shown that the magnitude of the in vivo polyclonal response was almost ten times lower than the in vitro response and that this could not be overcome by multiple PBA injections. This observation strongly suggests the existence of a natural suppressive mechanism, preventing excessive AA synthesis, which is absent in vitro. The suppressive immunoregulatory process is assumed to involve T dependent mechanisms, and indeed it has been shown that neonatal thymectomy considerably accelerated anti-DNA synthesis induced by PBA [69]. In summary, there are indications that a thymic regulatory process normally suppresses anti-DNA antibody production, perhaps via the non-specific T suppressor cells [69]. Thus, failure of the suppressor mechanism would lead to the expansion of autoreactive clones and to development of autoimmune disease; this is actually one of the accepted theories explaining the pathogenesis of autoimmune conditions such as SLE [53,92].

Another explanation for the development of overt autoimmune diseases is that the pool of autoreactive B cells normally present in healthy persons may be expanded and/or activated by polyclonal activators such as those produced by infectious agents (e.g. LPS, PG) [13]. Indeed, Fournie and co-workers have demonstrated that injection of bacterial LPS into normal mice

resulted in the release of DNA into the blood and in the formation of anti-DNA antibodies [64]. Supporting the view that infectious organisms induce autoantibody production are the findings of Welch et al. [8] that tetanus toxoid vaccination of normal adults generated a 2-3 fold rise in the frequency of IgM-RF levels. This mechanism can also explain the observed gradual increase in AA levels upon ageing; the increased autoantibody levels observed upon ageing could be the cumulative result of recurrent infections.

The idea that AA formation is a result of cross reaction between foreign and self determinants is not a new one. It is well known that antigen cross reaction is the accepted mechanism for the development of rheumatic carditis, and it has been suggested to be involved in the pathogenesis of ankylosing spondylitis, SLE and other autoimmune diseases [46,68]. Moreover, many infectious agents such as viruses, bacteria and parasites have been shown to enhance or even trigger the formation of AA's [10,73-76,93].

Still more convincing evidence for the existence of cross reactivity between anti-bacterial antibodies and autoantibodies was provided by Naperstek et al. [99]. They have demonstrated that certain Klebsiella-binding monoclonal Waldenström's macroglobulins shared idiotypic determinants with monoclonal lupus autoantibody, and these idiotypic determinants in the macroglobulins were shown to be closely related to their antigen-binding site. The determinant shared by the Waldenström's macroglobulins and the lupus monoclonal autoantibody is the 16/6 idio type. Moreover, Atkinson et al. [98] have shown by NMR-terminal amino acid sequencing that the primary structure of one Klebsiella-binding Waldenström's

macroglobulin (WEA) has a striking similarity to that of four lupus monoclonal autoantibodies. Likewise, Eilat et al. [96] have found marked homology between the amino acid sequence of the heavy chain of an NZB/NZW derived monoclonal antibody against dsDNA and that of anti-phosphorylcholine antibody from a CBA/J mouse directed against bacterial cell walls of certain bacterial species [97].

The concept that autoantibodies arise by cross reaction between self and non-self epitopes can be extended to explain the appearance of NAA's in normal individuals. Most studies indicate that the NAA antigen combining region is polyspecific, capable of binding a number of structurally related but not identical ligands [5,6,18,77]. Therefore, it is possible that the part of the B cell population which gives rise to AA's carries a polyspecific receptor capable of binding several different antigenic determinants. The fixation of a foreign antigen to this receptor would induce the B cell to undergo a series of divisions and mutations, which under the selective pressure of the antigen would lead to the production of a highly specific antibody for that antigen. Thus, natural polyspecific autoantibodies may constitute the antibodies secreted by these B cells prior to encountering foreign highly reactive antigens [5].

THE BIOLOGIC ROLE OF NAA'S

As previously stated autoreactive B cells represent the progeny of self specific B cell precursors, which arise during

ontogeny. Whatever their origin, the existence of such large numbers of autoreactive B cells, expressing a wide variety of specificities, leads to the conclusion that they probably have an important physiologic function. A summary of the important theories explaining the biologic role of NAA's is presented in table no. II.

Grabar [79] was the first to suggest a biologic role for NAA's in maintaining homeostasis. At a time when the concepts of "horror autotoxicus" and "forbidden clones" still prevailed, he suggested that autoantibodies are formed normally as a part of a physiological mechanism for cleansing the organism of self and non-self products. Grabar [79] formulated a theory stating that antibodies are transporters of metabolic and catabolic products; immunoglobulins reacting with metabolic substances are classical antibodies, while those reacting with catabolic products are autoantibodies. Therefore, Grabar views immunoglobulins not as a specific defense system, but rather as a physiological mechanism for clearing the organism of harmful self and non-self substances by opsonization, which subsequently leads to their phagocytosis and digestion. A good example of this mechanism was the finding of a new NAA reacting with alpha-galactosyl residues of human RBC membrane [9]. This AA, which was found in the serum of all healthy individuals studied, is believed to be important in the process of degradation of senescent erythrocytes.

An interesting hypothesis proposed by Cohen and Cooke [80] views NAA synthesis as the mechanism for achieving self tolerance. This seemingly paradoxical hypothesis states that NAA's displaying low affinity for self antigens may prevent autoreactive clones from reacting vigorously with self antigens by binding to these antigens and masking their antigenic

determinants. It is well known that self mimicking epitopes on foreign invading agents such as bacteria are capable of triggering vigorous autoimmune response. Therefore, the immune system must have ways of avoiding responses to self mimicking determinants while reacting with adjacent foreign structures. Cohen and Cooke suggest that NAA's act as a filter that enables only non-self antigens to induce a powerful immune response, while antigens masked by the pre-formed NAA's fail to elicit an aggressive response. Alternatively, NAA's may prevent autoreactive B cells from reacting with self antigens by blocking the receptors on these cells, thereby downregulating their own synthesis via the interaction with cell surface receptors [83]. The Cohen and Cooke hypothesis is very controversial, and Dziarski [94] challenged it by bringing experimental evidence which contradicts it. Dziarski [94] points to the well established phenomena that IgM antibodies can specifically enhance humoral immune responses when administered with or without their respective antigen, whereas IgG antibodies suppress these responses; since NAA's are primarily IgM, he claims that they would be expected to enhance rather than suppress the autoimmune responses in the host, and even may trigger the development of autoimmune disease. However, it should be emphasized that a substantial portion of NAA's are of the IgG class [2,5,9]. Moreover, in the studies of Ayransas and colleagues [5] it was found that 70% of the NAA's purified were IgG.

Others think that the role of NAA's is exactly the opposite, namely in triggering B cells to respond [19]. Indeed, as Dziarski points out [94], IgM antibodies, which constitute the majority of NAA's, can enhance humoral immune responses. This

hypothesis claims that contact with self antigens may be vital for priming the cellular immune system, a phenomenon which has been demonstrated for major histocompatibility antigens [91,92], but that can be extended to apply to any self antigen. While T cell self recognition may be restricted to HLA antigens, B cell reactivity, which may also be dependent on self recognition, can be mediated via NAA's. The theory can be further extended, and perhaps AA's play a role in any cell to cell recognition and interaction. However, there are no sufficient data to support this view.

The unraveling of the idiotypic-anti-idiotypic network, while shedding light on some of the mysteries of immunoregulation, created new puzzles concerning antibody and autoantibody synthesis. Several lines of evidence support the notion that anti-idiotypic antibodies play a regulatory role in the normal immune response. For example, anti-idiotypic production has been demonstrated during the response to TNP in both chicken and mice [55]. Actually, the idiotypic-anti-idiotypic reactions were the first immune processes shown to involve autoantibodies, serving to upregulate or downregulate immune responses. Although the idiotypic-anti-idiotypic responses still await elucidation, it has been suggested that NAA's are part of this complex network [36]. To date there are only a few reports on the participation of NAA's in idiotypic responses; however, the ongoing research into the function of the idiotypic network will certainly serve to elucidate the role of NAA's in these complex immune responses.

In conclusion, we propose a different function for NAA's, namely enhancing host immune reactions to foreign infectious agents. It is well known that B cell activation by T cells requires binding of T cell receptors to classical HLA antigens

on the B cell membrane. In a similar manner macrophage activation requires recognition of and binding to DR determinants on the T cell surface. These autoimmune responses are a prerequisite to any normal immune reaction without which foreign antigens could not be recognized, and therefore would not generate a normal immune response. We believe that NAA's are part of this system, functioning to strengthen immune responses to invading organisms. When, for example, a cell is infected by a virus, viral antigens are expressed on the cell surface. Antibodies formed against these viral membrane antigens bind to them, thereby inducing a complement reaction or opsonization and phagocytosis. We believe that pre-formed autoantibodies binding to self antigens on such an infected cell can enhance the reaction induced by the anti-viral antibodies. This can be achieved by improving the capping process on the cell membrane, or by inducing opsonization in conjunction with the classical anti-viral antibodies. NAA's may even be essential for macrophage and killer cell recognition of infected cells in exactly the same way that MHC determinants are necessary for T cell recognition. Indeed, natural antibody levels were found to correlate with host resistance and disease outcome [20,21]. However, the validity of our hypothesis can be assessed only by further studies on the participation of natural autoantibodies in immune responses.

CONCLUSION

There exists considerable evidence showing that normal humans and mice possess lymphocytes secreting antibodies reacting with a variety of self antigens. It is estimated that 10-30% of B

cells produce autoantibodies (80). Furthermore, even individuals with high levels of autoantibodies (e.g. relatives of patients with autoimmune diseases) may have no signs of autoimmune disease, making the line between disease state and normal state uncertain. These and other findings reviewed here illustrate that autoimmunity cannot be regarded solely as a pathological state. On the contrary, it appears that autoimmune reactions may be crucial to normal immune responsiveness. However, the puzzle of natural autoantibodies is only beginning to unravel, and it seems that we are examining only the tip of the iceberg in this complex autoreactive system. We believe that understanding the origin and function of NAA's will serve to clarify the pathogenesis of autoimmune diseases and it is hoped that new experiments will help answer the questions raised in this review.

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